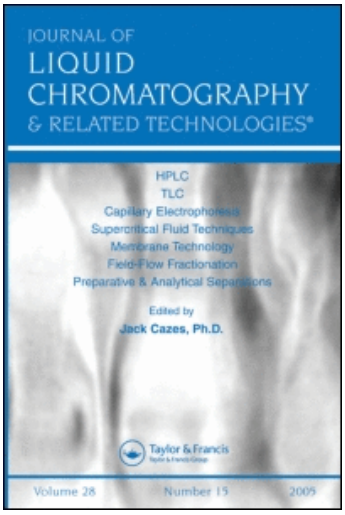

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Study of Various Treatments to Isolate Low Levels of Cider Proteins to Be Analyzed by Capillary Sieving Electrophoresis

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ABSTRACT

Four different sample treatments (ethanol precipitation, dialysis, ultrafiltration, and gel filtration) to isolate cider proteins were tested in this work. Purified cider proteins were analyzed by capillary sieving electrophoresis (CSE) using linear polyacrylamide as a sieving medium under optimized conditions; with this technique, separation, and molecular weight determination of proteins are possible. The electropherograms obtained in the protein analysis with each treatment were compared to choose the one that led to the best results. This was found to be ultrafiltration; many peaks were obtained in the electrophoretic profile and their spectra corresponded to a protein. Bradford analysis confirmed this choice. These results were compared with those obtained by sodium dodecyl sulfate

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polyacrylamide gel electrophoresis (SDS-PAGE), the molecular weights of the protein bands agreeing with the molecular weights of the electrophoretic peaks obtained with ultrafiltration.

Key Words: Cider proteins; Capillary sieving electrophoresis; Ethanol precipitation; Gel filtration; Dialysis; Ultrafiltration.

INTRODUCTION

Proteins play an important role in fermented beverages, they help to enhance foam characteristics in sparkling wines[1–3] and beer[4–6] thanks to their tensioactive properties,[7] and are also responsible for hazes and the formation of sediments.[8,9] On the other hand, they can become a valuable "finger print" for characterizing fermented beverages, since the protein fraction is not influenced by the nature of the soil or the climate, but is genetically defined. In fact, different authors have analyzed wine and must protein fractions in order to characterize them.[10–12]

As in other beverages, proteins play an important role in cider, a fermented beverage made from apple juice following traditional methods. The production of natural cider has become one of the major economic resources of Asturias (Spain). Hence, to make cider of a high quality is one of the objectives of local cider makers. Analysis of proteins is important to control the quality of the cider, since these affect the visual aspect of this beverage (foam behavior and haze formation) and enable geographical characterization. It is particularly useful to know their molecular weights, since some authors have demonstrated the importance of this parameter in foam constitution,[4,13] haze formation,[14] and characterization.[11]

Analysis and molecular mass determination of proteins is usually carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), but this technique is time consuming and quantification is difficult.[15] Capillary gel electrophoresis (CGE) and capillary sieving electrophoresis (CSE) have many advantages over the traditional slab gels and have been employed in recent years by many authors.[16–19] In the present study, CSE employing linear polyacrylamide has been used.

A purification step is needed before analysis, as cider is a complex matrix containing many substances that may interfere in the analysis of proteins. A concentration step is also needed, due to the low protein levels in this beverage (about 20 mg/L). There are different methods for isolating proteins; for example, M. Santoro[20] uses ethanol precipitation in wine samples. Ultrafiltration is another example employed in wines[9,14,19,21] and apple juices,[22] as it is a simple and effective system for separating substances with a high

molecular weight, like proteins, from the rest of the matrix. Dialysis is one of the most widely employed processes for purifying proteins[3,10–12,23,24] but it has a drawback, it is slow. It can take days to purify high volumes of liquid. Finally, gel filtration is a faster alternative to dialysis, being a rapid desalting system used by a number of authors.[25,26]

In this study, we compared four different methods (ethanol precipitation, ultrafiltration, dialysis, and gel filtration) to isolate cider proteins to be analyzed by CSE. The results obtained were compared with those achieved by SDS-PAGE.

EXPERIMENTAL

Reagents

Standard proteins, *tris*(hydroxymethyl)aminomethane (Tris), aspartic acid, (3-methacryl-oxipropyl)trimethoxysilane, and amberlite XAD-4 were obtained from Sigma (St. Louis, MO). Benzoic acid, sodium dodecyl sulfate (SDS), acrylamide, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and protein assay dye reagent concentrate were purchased from Bio-Rad (Hercules, CA). 2-mercaptoethanol, vegetable charcoal powder, and ethanol were supplied by Merck (Darmstadt, Germany). Urea and NaOH were obtained from Probus (Badalona, Spain). All were of analytical reagent-grade.

Milli-Q water (Millipore, Milford, MA) was used throughout. All the solutions were filtered through 0.45 μm with Millex-HV PVDF syringe filters and the cider samples were filtered through 0.22 μm Durapore® membrane filters from Millipore.

For the SDS-PAGE analysis, *N*, *N*′-methylene-*bis*-acrylamide, bromophenol blue, and dithiothreitol were obtained from Bio-Rad. Silver nitrate, sodium carbonate, methanol, glutaraldehyde, glycerol, and citric acid were purchased from Merck. Acetic acid and formaldehyde were supplied by Probus.

Materials

A Selecta 540 centrifuge (Barcelona, Spain) was used in sample treatment. Ultrafree® Centrifugal filter devices with a 5 kDa molecular weight cut off (MWCO) membrane and Amicon® Ultra filter devices with a 10 kDa MWCO membrane obtained from Millipore were employed; Spectra/Por dialysis membranes with 3.5 kDa and 5–6 kDa MWCO were supplied by

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Spectrum (Rancho Domínguez, CA); disposable PD-10 mini-columns of Sephadex® G-25 were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Instrumentation

A Hewlett-Packard (Waldbronn, Germany) ^{3D}CE apparatus equipped with a diode array detector and a HP ChemStation software package was used. Fused silica capillary tubes with 100 μm internal diameter were obtained from Tecknocroma (Barcelona, Spain).

Reversed polarity and electrokinetic injection (−10 kV, during 5 min) were used in the analysis of the SDS-protein complexes. Detection was performed at 220 nm.

A Power N Pac 300 electrophoresis instrument, from Bio-Rad, was used for the SDS-PAGE analysis and a Spectronic 20 spectrophotometer, supplied by Milton Roy (Rochester, NY), was employed for the Bradford protein assay.

Electrophoretic Conditions to Analyze the SDS–Protein Complex

Previously we had established the electrophoretic conditions for the analysis of cider proteins according to their molecular mass. The following are the optimized conditions: capillary dimensions were 32.5 cm (effective length, 24 cm) and 100 μm internal diameter; coating was performed employing the method described by Hjertén,[27] the capillary was rinsed with water (2 min) and then with the electrophoresis gel (5 min) between subsequent runs. Linear polyacrylamide was used in the electrophoresis gel and its composition was 4% (w/v) acrylamide, 0.19% (w/v) ammonium persulfate, 0.06% (w/v) TEMED, 0.1 (w/v) SDS, 50 mM Tris, and 35 mM aspartic acid. The applied voltage was 7 kV (reversed polarity), and the temperature was set at 25°C. The composition of the sample buffer was 1% (w/v) SDS, 50 mM Tris, and pH 8.9.

Before analysis, the five proteins used as standards were dissolved in 0.5 mL of water and in 0.5 mL of sample buffer. Benzoic acid and 2-mercaptoethanol were respectively added as internal standard and reducing agent to the protein mixture, which was then boiled for 10 min. The solution was filtered through 0.45 μm before analysis. A reagent blank was prepared mixing 0.5 mL of water with the rest of reagents, and without the protein standards. Cider concentrates were mixed with the sample buffer and then treated in the same way.

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Protein Molecular Weight Determination

The five proteins used as standards of known molecular mass were separated under the previously explained optimized conditions; their migration times were then measured and a calibration plot of the logarithm of molecular mass vs. the inverse of the relative migration time was constructed. This plot allows molecular weight determination of unknown proteins.

Sample Treatments

Cider is a complex matrix with very low concentrations of proteins, so it must be cleaned and concentrated before CE analysis. We used some common methods to isolate proteins from the selfsame cider to compare the obtained results.

Before sample treatment, the cider was kept in the ultrasonic bath for 5 min (to remove CO_2 bubbles and to liberate proteins) and was centrifuged at 2900*g* for 30 min (to remove solid particles). The procedure at the end of each treatment was similar to the one followed for the standard proteins.

Ethanol Precipitation

Cider was 25-fold concentrated in a rotary vacuum evaporator at 30°C. Proteins were recovered by precipitation, by adding 8 mL of ethanol to 2 mL of concentrate and centrifuging at 2900*g*. for 20 min. The precipitate was dried with N_2, and finally dissolved in 0.5 mL of water and 0.5 mL of sample buffer.

Dialysis

Cider was dialysed against Milli-Q water on 3.5 kDa and 6–8 kDa MWCO membranes, stirring at 4°C for 36 hr, the water being replaced every 12 hr. Then 50 or 100 mL were concentrated at 30°C in a rotary vacuum evaporator to 0.5 mL, this volume then being mixed with 0.5 mL of the sample buffer.

Ultrafiltration

It was necessary to filter the cider samples through 0.22 μm before ultrafiltration to avoid the membrane getting blocked. The cider was cleaned and concentrated by centrifuging 15 mL through a 5 or 10 kDa MWCO membrane until the volume of the retained suspension was 1 mL. Subsequently, 10 mL of

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water were added and it was then centrifuged again. This wash step was repeated, and finally, when the volume decreased to 0.15 mL, another 0.15 mL of sample buffer was added to the remaining solution, resulting in a 50-fold concentration.

Gel Filtration

It is well known that the gel filtration technique allows rapid desalting of samples. The procedure was as follows: 2.5 mL of cider filtered through 0.22 μm was added to a mini-column previously equilibrated with 25 mL (10 × 2.5 mL void volume) of water. Subsequently, elution of proteins was carried out with 3.5 mL of water. Six mini-columns were needed to clean all the cider, since the initial volume was 15 mL. Finally, the eluted volume from each column was concentrated in the rotary vacuum evaporator until it was dried, then 75 μL of water were added and the solution was mixed with the same volume of sample buffer (0.15 mL total volume), resulting in a 100-fold concentration.

Determination of Protein Concentrations

The Bradford protein assay[28] was employed to determine protein concentrations in cider before and after the sample treatments. All cider samples were filtered through 0.22 μm. The standard used was bovine serum albumin. The dye reagent (1 mL) was added to the cider (2 mL cider + 2 mL water) and protein samples (4 mL), and the absorbance at 595 nm was measured after it had stabilized (usually 10–15 min).

SDS-PAGE

SDS-PAGE was carried out according to the Laemmli method,[29] employing acrylamide concentrations of 12.3% T (acrylamide plus *bis*-acrylamide, w/v), 2.6% C (*bis*-acrylamide divided by acrylamide plus *bis*-acrylamide, w/w) for the resolving gel and 4% T, 2.6% C for the stacking gel. Sample concentration and purification is described in Paragraph 2.4. c. Urea of 9 M (200 μL) was added to 800 μL of concentrated cider from the initial 50 mL and were then centrifuged for 15 min at 13,000 *g*. Ethanol was added to the liquid (upper layer) and this was then centrifuged again. The precipitate was dissolved in 50 μL of sample buffer,[29] and 20 μL was introduced in the polyacrylamide gel after boiling for 5 min.

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Electrophoresis was performed at a constant voltage setting of 300 mV for the first 5 min and then 200 mV. Protein bands were visualized by the silver staining described by Morrisey.[30] Bio-Rad molecular weight standards were used as markers to determine the molecular mass of the SDS-proteins.

RESULTS AND DISCUSSION

Before the sample analysis, a reagent blank electropherogram was obtained. As can be seen in Fig. 1, there are not any peaks.

Ethanol Precipitation

The electropherogram shown in Fig. 2 presents some peaks isolated by precipitation at room temperature. Precipitation was also carried out at 4°C[31] with

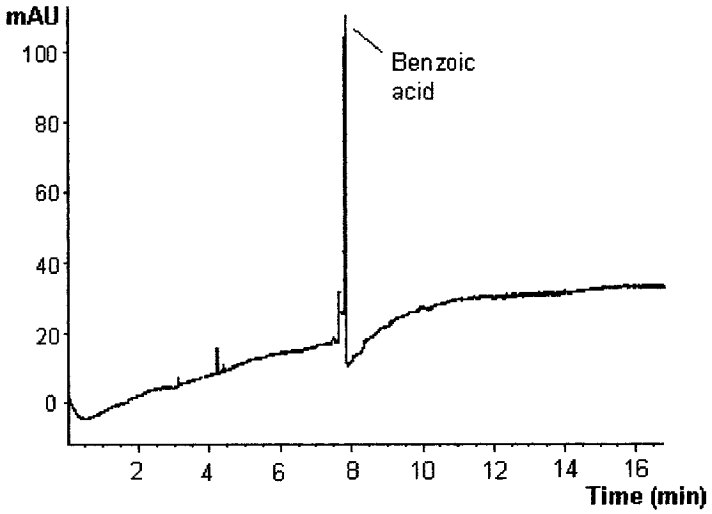

Figure 1. Electropherogram of a reagent blank, where benzoic acid has been added as an internal standard. Conditions: coated capillary, 100 μm internal diameter, 24 cm effective length, rinsed with water between subsequent runs; electrokinetic injection (−10 kV, 5 sec); temperature 25°C; detection wavelength 220 nm; electrophoresis gel Tris 0.05 M/aspartic acid 0.035 M, 0.1% SDS; voltage 7 kV (reversed polarity); current intensity 80 μA; the sample buffer used was 0.05 M Tris, 1% SDS, pH 8.9.

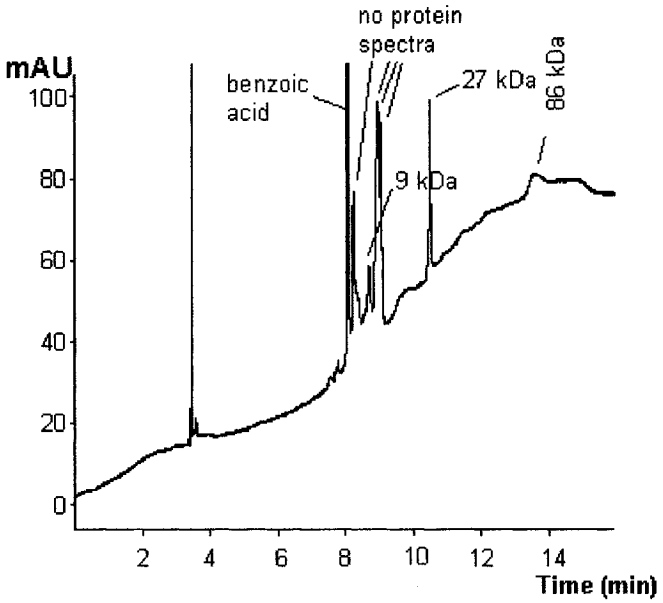

Figure 2. Electropherogram of a cider precipitated with ethanol. Conditions as in Fig. 1.

the aim of increasing the amount of precipitated protein and, subsequently, the peak area or the peak number, but no improvement was found.

Not only proteins are isolated with the applied treatment, since other compounds may precipitate with proteins. Hence, some peaks might not correspond to proteins; therefore their spectra were studied. Proteins usually present two maxima, one at 215 nm and the second one, much less intense, at 280 nm. Most of the peaks in Fig. 2 only present the 215 nm maximum. These peaks were consequently compared with the SDS-PAGE analysis and do not correspond to any protein band; accordingly, they are probably not proteins. Thus, this treatment is not effective for purifying cider proteins, because of the high viscosity of the obtained concentrate, probably due to the high concentration of sugars, which had not been removed with precipitation.

Dialysis

Two different MWCO were tested. Membranes of 6–8 kDa were chosen, since these could remove the more interfering compounds and there was not an appreciable loss in protein. The results obtained with this treatment can

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be seen in Fig. 3. The protein pattern presents many peaks, but there is a drift of the UV base line. This is due to substances other than proteins that absorb at the chosen wavelength. Peak spectra were examined and all of these showed two maxima, one at 215 nm and the other at 280 nm, similar to a protein spectrum.

To increase sensitivity, the cider was 100-fold concentrated. However, no improvement was found, since a stronger drift of the UV base line appeared and peaks could hardly be distinguished. This indicates that the dialysis step cannot effectively remove some substances that cause the drift.

Ultrafiltration

Preliminary studies demonstrated that Ultrafree® filters led to higher recoveries of proteins than Amicon® Ultra filters, hence, the latter were discarded and the Ultrafree® filters were used for rest of the studies.

Figure 4 shows the protein profile obtained with this treatment. As can be seen, there are a large number of peaks, ranging from approximately

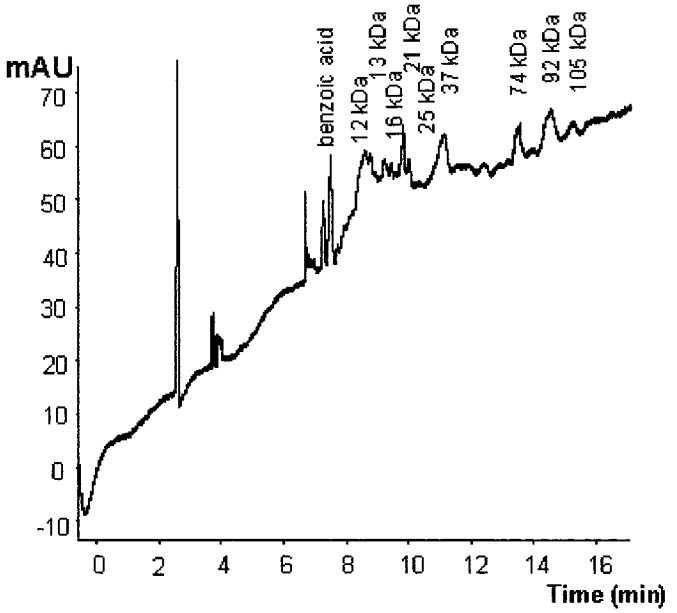

Figure 3. Electropherogram of a cider dialyzed against water with 6–8 kDa MWCO membranes. Conditions as in Fig. 1.

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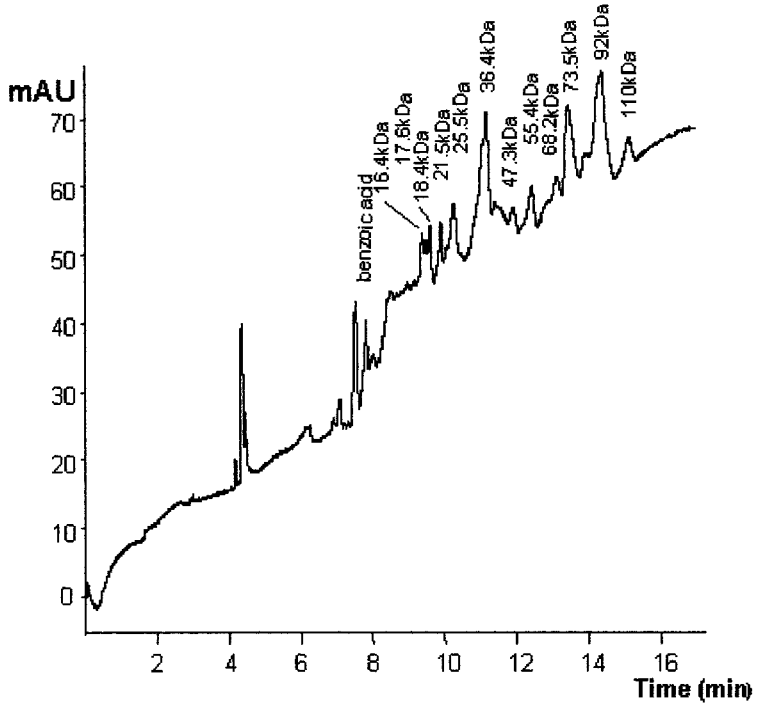

Figure 4. Electropherogram of a cider ultrafiltered with filters of 5 kDa MWCO and cleaned with water. Conditions as in Fig. 1.

16–110 kDa, that are higher than the peaks obtained by dialysis. Their spectra correspond to a protein, as Fig. 5 demonstrates, where the two maxima can be seen. Note that the peaks with 25.5, 36.4, 55.4, 73.5, and 92 kDa are higher than the rest. These correspond to major proteins, although minor proteins could play an important role in cider.

Although there are a large number of peaks in the electropherogram, this presents a drift of the UV base line. Attempts to decrease the drift by adding different compounds to the cider failed. Tris/HCl (10 mL) 20 mM with pH 9 were added instead of water in the first wash step, since a basic pH can prevent the strong interactions of proteins with polyphenols, thus eliminating these compounds. This change in the methodology resulted in a great increase in the drift and in a decrease in the peak areas in the electropherogram, due to the oxidation of the polyphenols that were not effectively eliminated.

Addition of urea, until a concentration in cider of 6 M, was tested. This was added before all the treatment steps to avoid loss of proteins in

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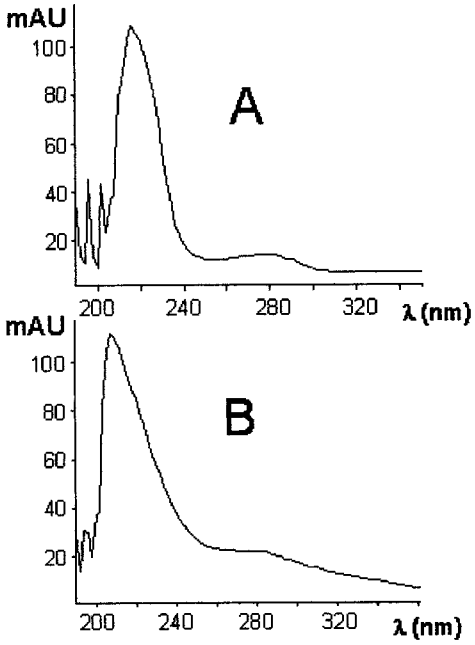

Figure 5. UV spectra of (A) the 10 min peak (21 kDa) from Fig. 3 and of (B) a protein used as standard (carbonic anhydrase).

centrifugation and filtration steps, since urea can dissolve proteins and it prevents interactions due to the proteins being denaturalized. However, urea did not show any improvement. SDS was also added until a concentration in cider of 300 mg/L. This compound has a similar effect; it dissolves proteins because it forms a complex with the protein, which decreases interactions with other compounds such as polyphenols. The addition of SDS had a similar effect, since neither polyphenols nor SDS could be eliminated.

Finally, adsorbent agents were added[32] before ultrafiltration (6 g of amberlite XAD-4 or 50 mg of charcoal activated powder were stirred with 30 mL of cider during 2 hr at room temperature). These only decreased the amount of protein, so the peak areas were smaller than those obtained without additives.

Once it was observed that the addition of the agents described above did not lead to any improvement, the repeatability and reproducibility of the method were measured. RSD data of the molecular weight found in the same cider, for the same and for different sample preparations, are listed in Table 1.

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Table 1. Repeatability and reproducibility data of the peak molecular weight, in kilo Daltons (kDa), found in an ultrafiltered cider (see Fig. 5).

Molecular weight (kDa)	RSD%	
	Repeatability[a] $n = 3$	Reproducibility[b] $n = 3$
16.4	3.7	3.8
17.6	3.8	3.9
18.4	3.6	3.6
21.5	3.5	3.6
25.5	3.3	4.3
36.4	2.9	3.0
47.3	2.1	2.1
55.4	2.3	5.3
68.2	2.0	2.6
73.5	2.5	3.1
92	1.9	2.0
110	2.3	2.0

[a]Inter-day repeatability data calculated with the same ultrafiltered sample.
[b]Inter-day reproducibility data calculated with three different ultrafiltered samples.

Gel Filtration

In spite of the good results obtained with ultrafiltration, this is a slow process that can take many hours. The gel filtration process is very similar to dialysis and ultrafiltration; it separates high molecular weight compounds from the rest of substances with lower molecular weights. However, gel filtration is much faster, it takes only few minutes. We, therefore, used this method to isolate cider proteins. However, protein recovery was very limited, as Fig. 6 shows. Despite analyzing 100-fold concentrated cider, only three small peaks can be seen in the electrophoretic profile. The drift of the UV base line has disappeared, but so have cider proteins. This treatment, as can be seen in Table 2, led to the lowest protein recovery and was, hence, discarded.

Protein Quantification

The Bradford assay has been used by several authors to quantify grape juice and wine proteins.[8,14,33] Samples are previously cleaned to separate

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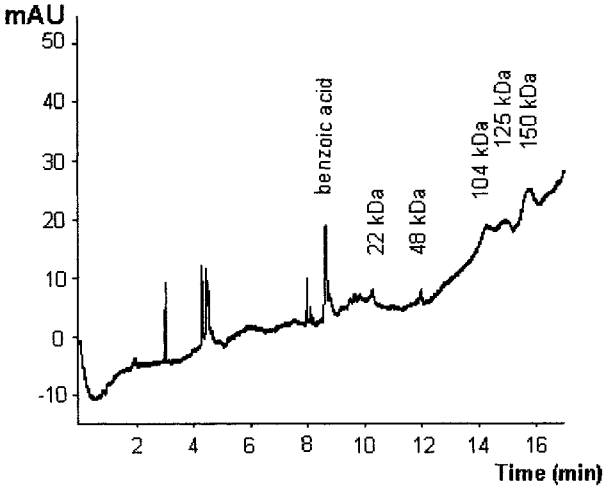

Figure 6. Electropherogram of a cider filtered through Sephadex G-25 and eluted with water. Conditions as in Fig. 1.

proteins from small amounts of peptides and polyphenols that would absorb at 595 nm, since they combine with the dye reagent.

The total soluble protein concentration was determined in samples before and after cleaning. The results can be seen in Table 2. Protein concentration in natural cider is higher than the concentration in treated cider, in accordance with the explanation given above. Ultrafiltration with Ultrafree filters is, for the moment, the preparation procedure that shows the highest protein concentration. This confirms the choice of this treatment as the best among the rest of the treatments studied.

Table 2. Concentration of proteins in cider before and after sample treatments as determined by the Bradford dye-binding assay. All cider samples were filtered through 0.22 μm.

Cider	Protein concentration (mg/L)
Natural cider	24.5
Precipitated and redissolute cider	7.3
Dialyzed cider (6–8 kDa)	11.0
Ultrafiltered cider (Amicon filter)	6.8
Ultrafiltered cider (Ultrafree filter)	19.0
Cider cleaned with Sephadex	2.6

SDS-PAGE

As stated above, ultrafiltration led to the best results. Spectra taken at the apex of the electrophoretic peaks indicate that they are probably proteins, but further demonstration can be achieved by SDS-PAGE, where the detection system stains only proteins. The molecular weights of proteins can also be estimated using this technique. The results can, hence, be compared with those achieved by CSE. Figure 7 demonstrates the agreement between the molecular weights obtained by CSE and SDS-PAGE.

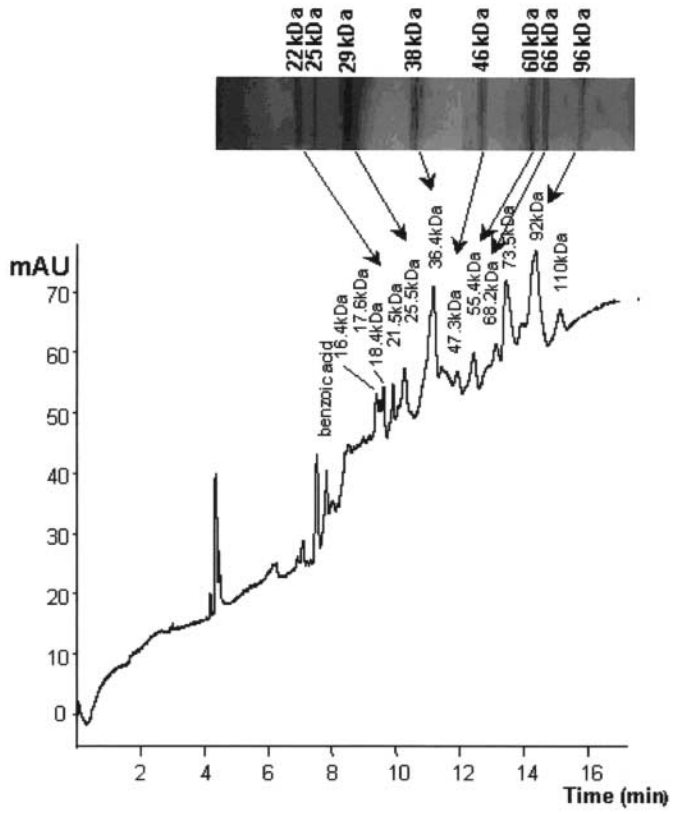

Figure 7. Comparison between the protein profile of an ultrafiltered cider obtained by capillary electrophoresis and the protein bands obtained by SDS-PAGE.

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CONCLUDING REMARKS

Of the four different processes studied in this work, ultrafiltration with a cleaning step was selected as the most effective. The obtained electropherogram demonstrates that this is a suitable sample treatment for isolating cider proteins, since, in spite of the drift of the UV baseline, there are many peaks in the protein profile. The results obtained by SDS-PAGE and with the Bradford Assay confirm this fact.

CSE is a very useful technique, since it allows molecular weight determination of unknown proteins. Many peaks were found in the protein pattern obtained and their molecular weights were calculated. These peaks range from 16 to 110 kDa, the most intense peaks being at 25.5, 36.4, 55.4, 73.5, and 92 kDa. Other, less intense peaks were also found that could play an important role in cider. The analysis of cider with different foam characteristics showed different protein profiles, not only in the amount of peaks but also in their area. These results suggest that ultrafiltration followed by CSE analysis is a good choice for characterizing cider proteins.

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